

# Assignment of 2D TOCSY Spectra of Lignins: the Role of Lignin Model Compounds

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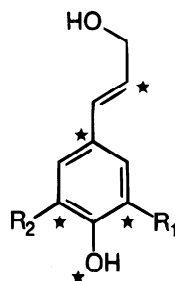
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The validity of using model compound data to facilitate the interpretation of solution-state two-dimensional TOCSY spectra of soluble wood lignins is demonstrated. The correspondence between model data and lignin correlations was such that it was possible to match the structure and stereochemistry of model compounds to structures present in the lignins by mapping model compound  $^1\text{H}$  chemical shift data to the cross peaks observed in the TOCSY spectra. For systematic comparisons of model data and lignin correlations, an  $XY$  scatter plot of model compound side-chain data was generated and then overlayed on the lignin TOCSY spectra. In addition to providing an accurate way of comparing model compound data with lignin correlations, this technique permitted the elucidation of some previously unassigned correlations. In situations where not all possible combinations of model compound side-chain stereochemistry and aromatic ring substitution were available, it was possible to generate internally consistent side-chain data from substituent effects. This allowed the prediction of the mode of attachment of certain inter-unit structures in the lignins.

KEY WORDS NMR;  $^1\text{H}$  NMR; 2D TOCSY; lignins; model compounds

## INTRODUCTION

Owing to its unique biosynthetic origin, it is not possible to give a unambiguous structural description of lignin. It has long been recognized that lignin is formed from the radical-initiated dehydrogenative polymerisation of (*E*)-4-hydroxycinnamyl alcohols (see Fig. 1), resulting in an achiral, polydisperse, random polymer.<sup>1</sup> A number of investigations of the key reactions involved in the biosynthesis of lignin have resulted in the postulation of a number of bonding schemes.<sup>1,2</sup> In



**Figure 1.** Lignin precursors: *p*-coumaryl alcohol ( $R_1 = R_2 = \text{H}$ ), coniferyl alcohol ( $R_1 = \text{OCH}_3$ ,  $R_2 = \text{H}$ ) and sinapyl alcohol ( $R_1 = R_2 = \text{OCH}_3$ ). Radical coupling takes place at the sites indicated by asterisks (except when the site is blocked by a methoxy group).

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general, there is little dispute about the structures and approximate relative abundances of major inter-unit linkages, although there is an increasing awareness that the distribution of structural units is not homogeneous in the cell wall.<sup>3-5</sup>

There has been a belated trend towards the use two-dimensional NMR methods for the study of the inter-unit structures present in lignins from woody plants and grasses. Although it is not possible to obtain the same degree of structural characterization as is possible with more NMR-friendly biopolymers such as peptides, nucleotides and oligosaccharides, we<sup>6-9</sup> and others<sup>10,11</sup> have shown that it is possible by the combined use of homo- or heteronuclear correlative techniques such as COSY, TOCSY,<sup>12</sup> HMQC<sup>13,14</sup> and HMBC<sup>15</sup> to determine unambiguously the presence or, in some cases, absence of inter-unit structures that have been previously characterized by degradative<sup>1,16</sup> and 1D NMR methods.<sup>17-21</sup> The recent application of the 3D HMQC-TOCSY experiment to poplar milled wood lignin (MWL) has shown that in the case of  $^{13}\text{C}$ -labelled lignins, considerable structural detail can be revealed.<sup>22</sup> The corollary to the use of solution-state NMR is, however, that the lignin sample must first be extracted from the cell wall. Yields of extracted wood lignins which can be solubilized before or after derivatization are low, and the degree to which these lignins are representative of the lignin *in vivo* is under current debate.<sup>21,23</sup> There is however, little dispute that milled wood lignin is the most representative carbohydrate-free lignin which can be studied by conventional spectroscopic methods.

Application of the second and third NMR dimensions to the structural analysis of lignin gives data resulting from entire side-chain spin systems, rather than relying on interpretations based around what are often single  $^{13}\text{C}$  chemical shifts or broad regions of  $^1\text{H}$  spectra, and it can also give new structural information that cannot be extracted from 1D NMR spectra. The complex, heterogeneous nature of lignin requires any NMR interpretation to be based around the use of appropriate model compounds for each of the structural units under investigation. The accuracy of the structural interpretation is thus critically dependent on the model compound being truly representative of the lignin unit, in terms of the aromatic substitution pattern, side-chain structure and side-chain stereochemistry.

It has previously been shown, using both dimers<sup>24,25</sup> and trimers,<sup>26</sup> that  $^1\text{H}$  NMR shifts in lignins are dictated by the bonded environment and are essentially independent of more remote structures and of the tertiary structure of the polymer. This insensitivity to through-space interactions, expected in  $^{13}\text{C}$  NMR, is not necessarily expected from  $^1\text{H}$  NMR. This paper presents a comparison of  $^1\text{H}$  chemical shifts of acetylated model compounds representing side-chain inter-unit and end-group structures, with the 2D TOCSY correlations observed in acetylated milled wood lignin from black spruce (*Picea mariana*) and radiata pine (*Pinus radiata*). These species are both softwoods (gymnosperms) which are characterized by having lignin biosynthesized almost exclusively from coniferyl alcohol. By generating an XY scatter plot of published side-chain  $^1\text{H}$  chemical shifts, and overlaying these scatter plots on the 2D NMR contour plots, it is shown that it is possible to match entire side-chain spin systems in model compounds with structures present in the lignin samples. It is also possible to match both structural and stereochemical variations in model compound data for the predominant  $\beta$ -O-4 and  $\beta$ -5 structure to correlations observed in lignin. The use of this technique has also resulted in the elucidation of previously unassigned correlations from oxidised structures.

By generating substituent and stereochemical effects from the existing data for the  $\beta$ -1 (diarylpropane) structure, it was possible to develop a complete set of  $^1\text{H}$  NMR data for all possible structural and stereochemical combinations. In the case of the  $\beta$ -1 structure, these

data have allowed the prediction of the mode of attachment of these units in the lignin.

## RESULTS AND DISCUSSION

Lignin model compounds have played a key role in the determination of both the structure of lignins from woody plants and grasses and the reactions of lignin during chemical and mechanical processing of lignocellulosic biomass (in particular wood pulping). A wide range of model compounds have been synthesized, which more or less represent certain structural features of the lignin under investigation. A database of these compounds, which contains structural and NMR data for a wide range of lignin model compounds, has recently been developed.<sup>27</sup> To facilitate interpretation of lignin spectra, lignins are often acetylated to improve both solubility in common NMR solvents and dispersion of the side-chain chemical shifts, hence the model compounds must also be acetylated if they are to be used as interpretative aids.

Figure 2 shows the structures of a range of acetylated lignin model compounds representing the majority of inter-unit structures present, or proposed to be present, in lignins. The side-chain chemical shifts for these compounds, in chloroform-*d*, are presented in Table 1. In some structures, not all combinations of side-chain stereochemistry and aromatic substitution are published. In the case of structures **3c**, **4b-d** and **8a** and **b**, the data were obtained by developing substituent and stereochemical effects from available published data for related compounds. As an example, the calculated substituent effects for  $\beta$ -1 model compounds **3** and **4** are given in Table 2. The substituent effects account for the *erythro/threo* diastereoisomerism of the side-chain and the substitution of C-4 on the aromatic rings (acetoxy or alkoxy). Compounds **10-15** are representative of possible side-chain end-groups.

For the purposes of this investigation, which was focused on the side-chain structures in lignin, dimeric model compounds have been used to represent inter-unit structures. Although some trimeric models have been extensively characterised by NMR,<sup>26</sup> and are inherently more appropriate lignin models, the spectra were acquired in acetone-*d*<sub>6</sub>. Chloroform-*d* was chosen

Table 1. Side-chain  $^1\text{H}$  NMR chemical shifts for model compounds 1-15<sup>a</sup>

H	1a <sup>24</sup>	1b <sup>24</sup>	2a <sup>24</sup>	2b <sup>24,25</sup>	3a <sup>29</sup>	3b <sup>29</sup>	3c	3d	4a <sup>29</sup>	4b	4c	4d	5a <sup>31</sup>	5b <sup>31</sup>
H $\alpha$	6.12	6.08	6.08	6.02	6.10	6.08	6.07	6.05	5.98	5.96	5.95	5.93	5.51	5.46
H $\beta$	4.63	4.65	4.67	4.70	3.38	3.33	3.40	3.35	3.42	3.37	3.44	3.39	3.76	3.76
H $\gamma_1$	4.31	4.28	4.46	4.42	4.39	4.34	4.33	4.28	4.53	4.48	4.47	4.42	4.45	4.44
H $\gamma_2$	4.06	4.01	4.25	4.23	4.18	4.13	4.16	4.11	4.36	4.31	4.34	4.29	4.29	4.31
	6a <sup>31</sup>	6b <sup>31</sup>	7a <sup>32</sup>	7b	8a <sup>32</sup>	8b <sup>32</sup>	9 <sup>32</sup>	10 <sup>17</sup>	11 <sup>32</sup>	12 <sup>32</sup>	13 <sup>11</sup>	14 <sup>32</sup>	15 <sup>17</sup>	
H $\alpha$	4.8	4.76	5.47	5.42	5.43	5.38	—	6.60	5.90	5.93	2.67	—	7.42	
H $\beta$	3.1	3.11	4.77	4.77	4.69	4.71	5.62	6.16	5.43	5.40	1.96	4.52	6.62	
H $\gamma_1$	4.28	4.26	4.46	4.47	4.65	4.65	4.67	4.71	4.24	4.23	4.11	3.28	9.67	
H $\gamma_2$	3.94	3.91	4.13	4.14	4.55	4.55	4.52	—	—	—	—	—	—	

<sup>a</sup> Calculated chemical shifts are in italics.

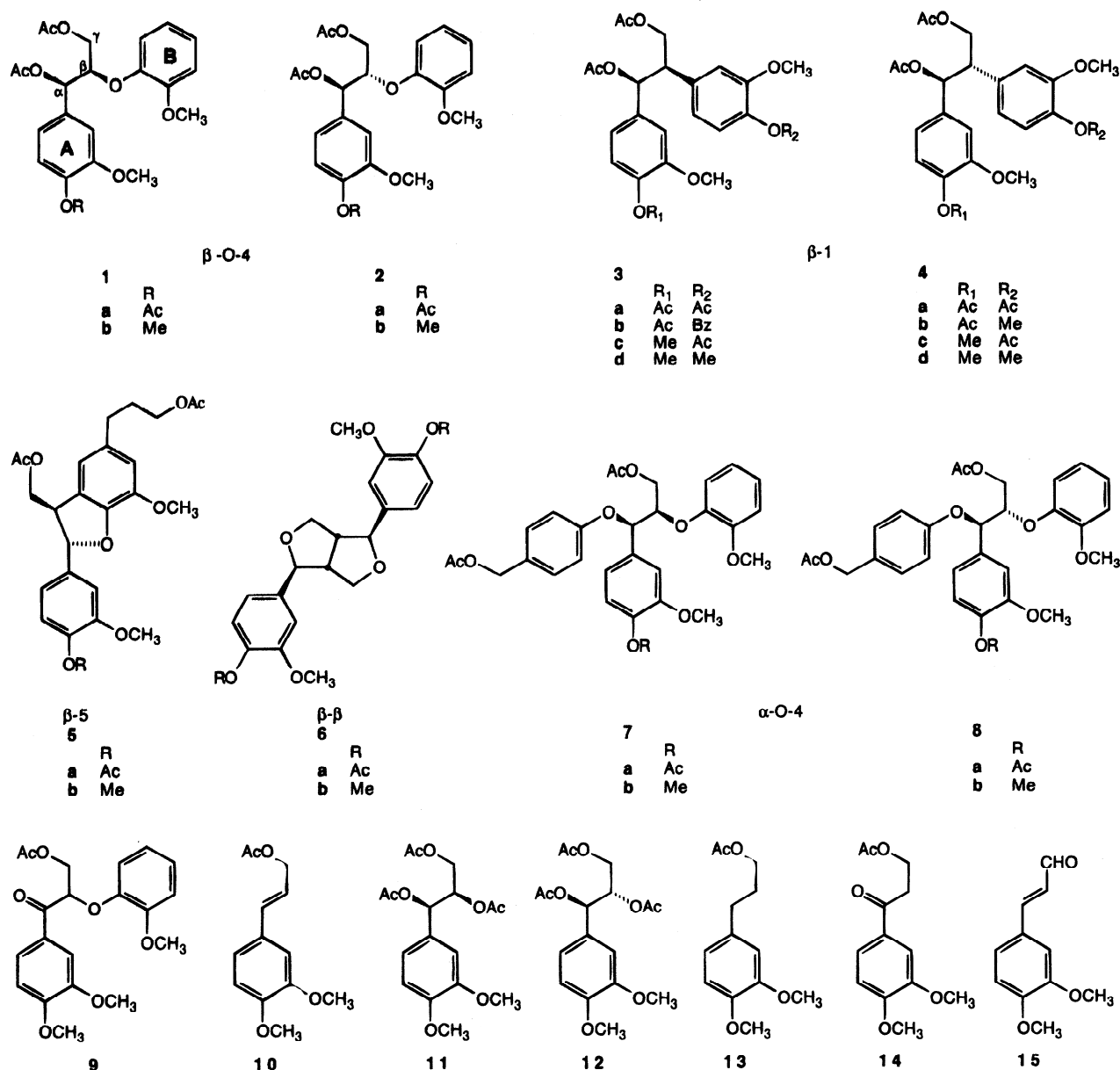


Figure 2. Acetylated lignin model compounds.

as the solvent for this study as there is a greater structural variety of acetylated model compounds whose chemical shifts are reported.

### Acquisition of TOCSY spectra

Although the HMQC technique has been shown to be of some value for the characterization of lignins, the 2D TOCSY experiment gives more structural information,

in terms of side-chain spin systems. There are a number of factors which should be taken into account when acquiring TOCSY spectra of lignins. As the lignins are polymeric, with number molecular weights estimated to be in the region of 10–20 kDa, with high polydispersity,<sup>33</sup> and transverse relaxation times estimated to be *ca.* 100 ms,<sup>7</sup> acquisition and repetition times should be kept short to maximize acquisition efficiency. The presence of strong, comparatively broad singlet signals from methoxy and acetoxy groups and weak, broad multiplet signals from the side-chain protons leads to a requirement for flat baseplanes and low *t*<sub>1</sub> noise ridges. It was found during the course of this investigation that the setting of the first data-point multiplier during processing was crucial for the production of baseplanes with no d.c. offset. Once the d.c. offset had been removed, it was possible to use a standard quadratic polynomial effectively to remove a residual baseplane tilt and to help reduce the intensity of the broad *t*<sub>1</sub> ridges associated with the methoxy and acetoxy signals.

Table 2. Substituent effects for β-1 model compounds 3 and 4

H	Δδ (ppm)		
	erythro → threo	A-ring acetoxy → A ring alkoxy	B-ring acetoxy → B ring alkoxy
α	−0.12	−0.03	−0.02
β	0.04	0.02	−0.05
γ <sub>1</sub>	0.14	−0.06	−0.05
γ <sub>2</sub>	0.18	−0.02	−0.05

### Interpretation of TOCSY spectra

The majority of side-chain correlations are observed in the  $\delta$  6.6–3 region of the TOCSY spectra of acetylated MWL (see Fig. 3). There are two sets of correlations observed outside this region resulting from arylpropanol **13** and coniferaldehyde **15** end-groups. Although there are a number of correlations shown in Fig. 3, the discussion in this case will be limited to the regions encompassed by the boxes in the figure. The expanded regions were taken as  $F_1$  slices and diagonally reflected to give Figs 5–7, as the  $F_1$  slices had better  $F_2$  digital resolution of the closely spaced  $H\alpha$  chemical shifts.

In order to provide an accurate and unambiguous method for the transcription of model compound chemical shift data on to the contour plots of the 2D spectra, a software-based method was developed. This was achieved by using graphing software to generate XY scatter plots of the model compound data and overlaying the scatter plot on the contour plot. The entire process could be carried out without the need to produce hard copies of the spectra. The graphing software (Deltagraph Pro) used could produce scatter plot 'spectra' of any desired  $F_2 \times F_1$  region with defined dimensions. The scatter plots could then be copied and pasted on to TOCSY contour plots in the drawing software (MacDraw Pro). The overlay of the two could then be achieved by positioning the scatter plot so that its borders overlapped the borders of the contour plot. The correlations points could then be edited within MacDraw Pro to allow them to be displayed as com-

pound numbers, for example. The expansions of the spectral regions discussed below were all generated in this fashion. An example scatter plot of all the model data from Table 2, replicating a long spin-lock TOCSY experiment, is shown in Fig. 4.

Figure 5 shows an expansion of region 1 of the extended spin-lock (112 ms) TOCSY spectrum of acetylated black spruce MWL. The numbers represent the positions of correlations in model compound structures, as generated from the scatter plot. The dominant correlation in this region contains the cross peaks arising from  $H\alpha$  to  $H\beta$ – $H\gamma_1$ – $H\gamma_2$  magnetization transfer in  $\beta$ -O-4-aryl ether structures of type **1** and **2**. The  $H\alpha$ – $H\beta$  correlations in this structure are surrounded with a cross-hatched box. There is an extremely good coincidence of the model compound chemical shifts with the topology of the contours. It is possible to identify the correlations arising from the four combinations of  $\beta$ -O-4 structural unit in terms of the 4-O substitution in the A-ring (i.e. acetylated or etherified) and side-chain stereochemistry (i.e. *threo* or *erythro*). This study complements fairly well a previous 2D  $J$ -resolved NMR investigation of Norway spruce MWL, which was able to show clearly the presence of all four  $H\alpha$  doublets from structural types **1** and **2**.<sup>6</sup> The correlations enclosed by the dotted box in Fig. 5 have until now been unassigned. These appear to be from  $H\alpha$ – $H\beta$  correlations in structures of type **1** and **2** which have a downfield shift for  $H\beta$  of ca. 0.2 ppm. This shift is consistent with the presence of an electron-withdrawing substituent on the B-ring. Although there is a dearth of

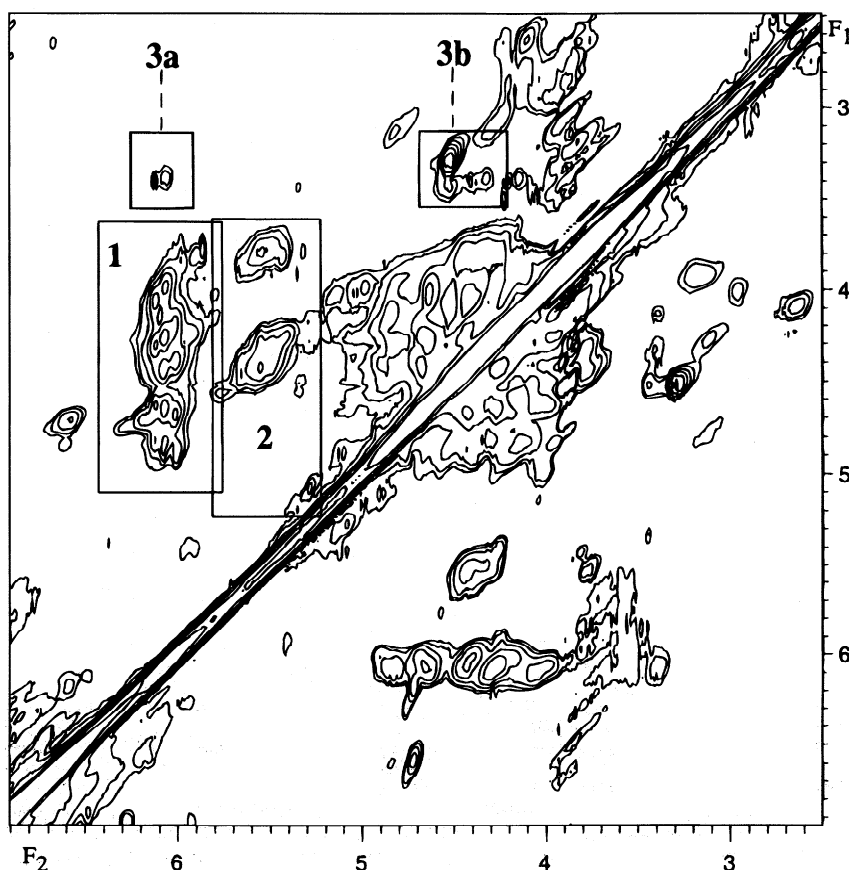
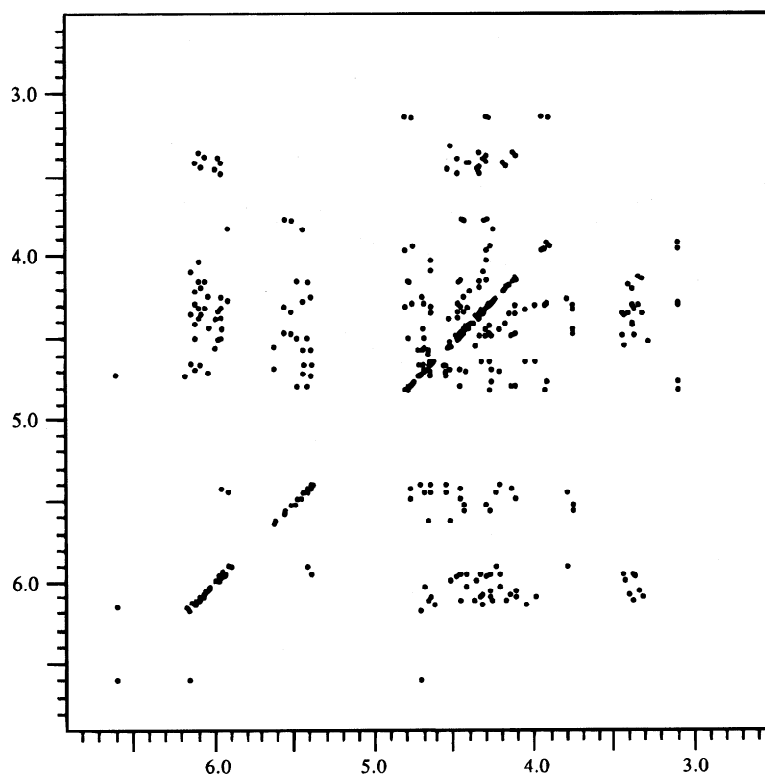


Figure 3. Side-chain region of TOCSY spectrum (spin-lock = 112 ms) of acetylated black spruce MWL.



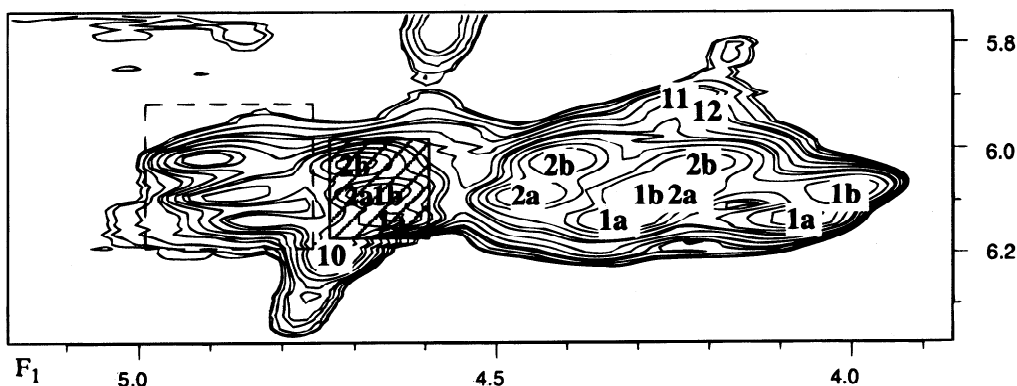
**Figure 4.** Scatter plot of model compound data presented in Table 1. The data are presented to replicate a long spin-lock TOCSY spectrum as shown in Fig. 3.

model compound data for structures of this type, it would be likely that  $\beta$ -O-4 structures which were etherified at the  $\beta$ -position with structures such as **9**, **14** and **15** would have a downfield shift for  $H\beta$  similar to that observed in the lignin correlations. We are currently synthesizing model compounds of this type, and the results of this investigation will be reported separately.

The  $H\alpha$ - $H\gamma$  correlations from arylglycerol end-groups **11** and **12** and the  $H\beta$ - $H\gamma$  correlation from coniferyl alcohol end-groups **10** are also seen in region 1. Structures of type **11** and **12** have recently been found in Norway spruce<sup>6</sup> and Scots pine lignins.<sup>8</sup> The biosynthetic origin of these structures has yet to be determined.

Region 2 of a TOCSY spectrum (spin-lock = 32 ms) of acetylated black spruce MWL, shown in Fig. 6, is dominated by the correlations from the  $\beta$ -5

(phenylcoumaran) structures **5**. Even though this is a short spin-lock experiment, both the direct  $H\alpha$ - $H\beta$  (cross-hatched) and relayed  $H\alpha$ - $H\beta$ - $H\gamma_1$ - $H\gamma_2$  correlations are visible. The positions of the model compound shifts for structures **5** appear to be offset from the corresponding lignin correlations by ca.  $-0.05$  ppm in  $F_2$ . The correlations for models of the non-cyclic benzyl 1,2-diaryl ( $\alpha$ -O-4) ether structural unit (structures **7** and **8**) are also shown in Fig. 6. These structures have been proposed from indirect chemical evidence to be important reactive linkages in lignins. However, we have recently shown that this type of inter-unit structure is not present in *Pinus radiata* MWL at NMR-detectable levels.<sup>34</sup> It is also clear from Fig. 6 that these structures are not detectable in black spruce MWL, as the model compound data are not in the vicinity of any lignin correlations attributable to the  $\alpha$ -O-4 structure.



**Figure 5.** Region 1 of spectrum in Fig. 3. Numbers represent side-chain correlations from model compounds. The  $H\alpha$ - $H\beta$  correlations from structures **1** and **2** are enclosed in the cross-hatched box. For correlations enclosed in dotted box, see text.



Correlations from  $\beta$ - $\gamma_1$  and  $\beta$ - $\gamma_2$  magnetization transfer in  $\beta$ -O-4 structures containing  $\alpha$ -keto groups (structure 9) are also observed in region 2. It is not certain whether these structures are naturally present in the lignins, or if they are formed by oxidative processes during or after isolation. These structures are generally not detectable in 1D  $^1\text{H}$  or  $^{13}\text{C}$  experiments owing to low sensitivity and signal overlap.

Figure 7 shows regions 3a and 3b of the TOCSY spectrum (spin-lock = 32 ms) of acetylated acetone-water-extracted *Pinus radiata* MWL. This lignin extract has been shown to be higher in  $\beta$ -1 structural units than the corresponding dioxane-water-extracted MWLs,<sup>35</sup> and the majority of the correlations observed in region 3 arise from the  $\beta$ -1 structure. The correlations in region 3a arise from  $\text{H}\alpha$ - $\text{H}\beta$  magnetization transfer, while the majority of the correlations in region 3b arise from  $\text{H}\beta$ - $\text{H}\gamma_1$ - $\text{H}\gamma_2$  magnetization transfer. The best correspondence of model compound data and lignin correlations is seen for structures 3a, 3c, 4a and 4c, suggesting that for MWL, the  $\beta$ -1 structural unit is predominantly phenolic at the B-ring. The expected chemical shifts for compounds 3c and 4c correspond more closely to the lignin correlations than do the expected correlations from structures 3a and 4a. It is also likely that, since structures 3a and 4a are phenolic at both the A- and B-rings (and correspond to either isolated  $\beta$ -1 dimers, or phenolic  $\beta$ -1 units which are linked via the A- or B-ring aromatic carbons), the major linkage of the  $\beta$ -1 structure is via an etherified A-ring, corresponding to model compounds 3c and 4c. These results must be interpreted with some caution, however, as appropriate model compounds for structures 3c and 4b-d are yet to be synthesized to confirm the predicted side-chain chemical shifts. The above result is at odds with a previous acidolysis investigation which suggested that  $\beta$ -1 units were attached to spruce lignin via an acid-labile B-ring linkage, with the A-ring phenol being free.<sup>36</sup> It should be noted, however, that this investigation was based on observation of recovered degradation products, and that it was not possible to determine the extent to which the yields of isolated products reflected the total content of the  $\beta$ -1 structures in lignin.

There is an extremely good match between model 14 data and the lignin correlation at  $\delta$  4.52/3.28 in region 3b. The fact that only one TOCSY correlation ( $\text{H}\beta$ - $\text{H}\gamma$ ) is available to characterize this end-group structure makes its assignment tenuous, although its presence is justifiable, as it would be expected to be formed from photochemically induced cleavage of A-ring etherified  $\beta$ -O-4 units by the mechanism of Schmidt and

Heitner.<sup>37</sup> The use of the TOCSY experiment for observation of structures of these types, which are difficult to observe by other means, may be of use in studies directed towards determining rates and mechanisms of photodegradation of wood.

## EXPERIMENTAL

Milled wood lignin (MWL) was prepared from *Pinus radiata* sapwood using acetone-water (9:1) as the extracting solvent.<sup>38,39</sup> Lignin NMR spectra were acquired in chloroform-*d* solutions (30–100 mg of sample in *ca.* 0.4 ml of solvent) at 300 K on a Bruker AMX360 spectrometer using a 5 mm conventional geometry four-nucleus (QNP) probe. Lignin chemical shifts were referenced to internal TMS. TOCSY spectra were acquired over a 9.5 ppm window in both  $F_2$  and  $F_1$ , with MLEV-17 spin-lock lengths ranging from 32 to 112 ms using the Bruker pulse program mlevtp. The short spin-lock spectra were acquired with 64 scans per  $t_1$  increment, the long spin-lock experiments used 128 scans per  $t_1$  increment. In all cases the  $2\text{K} \times 256$  increments were acquired with simultaneous data acquisition. After  $F_1$  zero-filling, TPPI Fourier transformation and squared cosine-bell apodization, the transformed data matrix was  $1024 (F_2) \times 256 (F_1)$  real points. Baseplane distortions were reduced by optimization of the first data-point multiplier in both  $F_2$  and  $F_1$  prior to transformation, which removed the d.c. offset in the baseplane. The residual  $F_1$  baseplane tilt was removed with a quadratic polynomial correction applied after transformation.

The model compound XY scatter plots were generated using DeltaGraph Professional (Deltapoint) and overlayed on spectra which had been imported into MacDraw Pro (Claris) by conversion of HPGL plot files using PlotView (Stevens Creek Software). All software was run on Apple Macintosh computers.

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